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Rapid Responses and Mechanism of Action for Low-Dose Bisphenol S on *ex Vivo* Rat Hearts and Isolated Myocytes: Evidence of Female-Specific Proarrhythmic Effects

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Running title: BPS promotes arrhythmias in female heart

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Abstract

Background: Bisphenol S (BPS) is increasingly been used as a substitute for BPA in some "bisphenol A (BPA)-free" consumer goods and in thermal papers. Wide human exposure to BPS has been reported; however, the biological and potential toxic effects of BPS are poorly understood.

Objective: To elucidate the sex-specific rapid impact of BPS in rat hearts and its underlying mechanism.

Methods: Rapid effects of BPS in rat hearts were examined using electrophysiology, confocal and conventional fluorescence imaging, and immunoblot.

Results: In female rat hearts, acute exposure to 10^{-9} M BPS increased heart rate and in the presence of catecholamine-induced stress condition, markedly increased the frequency of ventricular arrhythmia events. BPS increased the incidence of arrhythmogenic triggered activities in female ventricular myocytes, and altered myocyte Ca^{2+} handling, particularly spontaneous Ca^{2+} release from the sarcoplasmic reticulum. The dose responses of BPS' actions were inverted-U shaped. The impact of BPS on myocyte Ca^{2+} handling was mediated by estrogen receptor β signaling and rapid increases in the phosphorylation of key Ca^{2+} handling proteins including ryanodine receptor and phospholamban. The pro-arrhythmic effects of BPS were female-specific; male rat hearts were not affected by BPS at the organ, myocyte and protein levels.

Conclusion: Rapid exposure to low-dose BPS has pro-arrhythmic impact on female rat hearts; these effects at the organ, cellular and molecular levels are remarkably similar to those reported for BPA. Evaluation of the bioactivity and safety of BPS and other BPA analogs is necessary before they are used as BPA alternatives in consumer products.

Introduction

Bisphenols are a group of chemicals with two hydroxyphenyl functionalities in the structure (Liao and Kannan 2013). Bisphenols are widely used in the manufacture industry, with bisphenol A (BPA) being the most commonly used member in the production of polycarbonate plastics and epoxy resins. Examples of BPA-based products include food containers, baby bottles, beverage and food can lining, receipt thermal paper, and water pipes. There is broad human exposure to BPA. Detectable level of BPA is found in urine and blood in over 90% of individuals examined in various sample populations (Vandenberg et al. 2007; Vandenberg et al. 2012a; Ye et al. 2008).

BPA is an estrogenic endocrine disrupting chemical (EDC). A large body of evidence has linked BPA exposure to human diseases such as cancer, diabetes, obesity and various disorders in reproductive, neuronal, immune and cardiovascular (CV) systems (Diamanti-Kandarakis et al. 2009; Melzer et al. 2010; Zoeller et al. 2012). Due to the potential adverse health impact of BPA exposure, steps have been taken to reduce its usage in consumer products in recent years. European Union and U.S Food and Drug Administration have banned BPA's usage in baby bottles (European Commission 2011; US Food and Drug Administration 2012), and France has issued a ban of manufacturing and sale of all food packaging containing BPA starting in 2015 (Legifrance.gouv.fr 2012).

Bisphenol S (BPS, 4,4'-Sulfonyldiphenol, CAS 80-09-1) is composed of two phenol groups on each side of a sulfonyl group, similar to BPA (Fig. 1). BPS is increasingly been used as a

substitute for BPA in some "BPA-free" consumer products. BPS is also found in thermal papers including receipts, envelopes and airlines boarding passes (Liao et al. 2012b). Though more heat-stable and sunlight-resistant than BPA, BPS still leaches from food cans and containers under normal usage (Vinas et al. 2010). Human exposure to BPS has been reported and appears to be wide-spread. In a recent study, 315 urine samples were collected in US and seven Asian countries, from both females and males ranging 2 to 84 years old. BPS has been detected in 81% of the samples with an overall mean urinary concentration of 0.65 ng/ml or 2.6 nM (Liao et al. 2012a). Similar to BPA, BPS has been found to have estrogenic activities (Grignard et al. 2012; Hashimoto et al. 2001). While the health impact of BPA has been extensively studied, current knowledge on the potential biological effects and health impact of BPS is very limited.

Multiple epidemiological studies have showed that in adults, BPA exposure levels are associated with CV diseases or CV disease risk factors (Lang et al. 2008; Melzer et al. 2010; Melzer et al. 2012). However, a lack of association between BPA exposure and CV disease was reported by LaKind et al (LaKind et al. 2012). Previously we demonstrated that low-dose BPA rapidly promoted arrhythmogenic triggered activities in female rodent ventricular myocytes (Yan et al. 2011). Further, under stress or ischemic conditions BPA increased the risk for cardiac arrhythmias in the female rat hearts (Yan et al. 2011; Yan et al. 2013). These studies suggest a potential adverse impact of BPA on the cardiovascular system. The potential cardiac toxicity of BPS is entirely unknown. In the present study, the rapid impact of low-dose BPS on rodent

hearts and cardiac myocytes were investigated, with a focus on the arrhythmogenic effect of BPS and its underlying cellular and molecular mechanisms.

Materials and Methods

Reagents

Bisphenol S (BPS or 4,4′-Sulfonyldiphenol), CAS 80-09-1 was from Sigma-Aldrich. Bisphenol A (BPA), CAS 80-05-7 was from TCI America, lot 111909 (ground by Battelle), and was provided by the Division of the National Toxicology Program (DNTP) at the National Institute of Health/National Institute of Environmental Health Sciences.

Methyl-piperidino-pyrazole,1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP), CAS 289726-02-9;

2,3-bis(4-hydroxyphenyl)-propionitrile diarylpropionitrile (DPN), CAS 1428-67-7; and

4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP), CAS 805239-56-9 were from Tocris Bioscience. Other chemicals were from Sigma-Aldrich unless otherwise stated. BPS and BPA were dissolved in DMSO as stock solutions (0.1 M), and were stored in -20 oC. Experimental solutions were prepared fresh using glass containers. All experimental apparatuses were free of polycarbonate plastic to prevent possible leaking of bisphenols into the solutions. The effect of vehicle (DMSO) at the highest concentration (~10-4 M) used in the experiments have been tested, and were found to have no detectable effect on any of the endpoints examined in the study.

Animals

Adult Sprague-Dawley rats (200-250 g; Charles River) were used as non-surviving sources of whole heart preparations and isolated ventricular myocytes. Animals were maintained at two per cage in standard polycarbonate shoebox caging with Sani-chip bedding (Irradiated Aspen Sani-chip; P.J. Murphy Forest Products Corp.) to eliminate possible corn-based mycoestrogen exposure, and were fed ad libitum Teklad diet 2020 (Harlan Laboratories Inc.) which lacks soybean meal, alfalfa or animal products that may introduce uncontrolled levels of estrogenic compounds. Animal room conditions included a 14 hr light, 10 hr dark cycle, with lights on at 6 AM and off at 8 PM, and 60% humidity at 24 ^{o}C. Newly arrived animals were housed in the animal facility for at least two weeks before surgeries allowing for accommodation. Sterile drinking water was generated by a dedicated water purification system (Millipore Rios 16 with ELIX UV/Progard 2) that reduces oxidizable organics to less than 1% of source levels. Glass bottles were used to dispense water. All animal procedures were performed in accordance with protocols approved by the University of Cincinnati Institutional Animal Care and Use Committee and followed recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Animals were treated humanely and with regard to alleviation of suffering. A total of 75 animals were randomly allocated to experimental groups in this study, including 45 females and 30 males. These numbers, and those stated below for individual experiments, do not include animals used in procedures or experiments that were unsuccessful. Experiments involving surface electrocardiography measurements (see below) were performed

throughout the regular business day, between 9 AM and 6 PM. For all other experiments, surgeries (i.e., excision of the heart) were performed in the morning between 9 AM and 12 PM and assessments of the cardiac myocytes were performed during the rest of the business day. All procedures were performed at the approved satellite animal stations located in the laboratory. Animals used in this study were not previously used for other purposes.

Surface electrocardiography (ECG)

Rats were anesthetized with sodium pentobarbital (80 mg/kg, i.p.). The hearts were rapidly excised, cannulated via the aorta, and perfused on a Langendorff apparatus with Krebs-Henseleit solution as previously described (Yan et al. 2011). The solution contained (mM) NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, EDTA 0.5, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 11, pH = 7.4, bubbled with 95% O_2 and 5% CO_2. Hearts were perfused at a pressure of 80 mmHg and perfusion rate of ~15 ml/min. Surface electrocardiograph was continuously recorded from the heart, with two electrodes positioned at the base and apex of surface of the heart. Data collection and analysis were performed using the Powlab 4/30 data acquisition system and LabChart 7 software (AD Instruments). Treatment chemicals were included in the perfusate following at least 1 hour of stabilization under control condition; hearts were exposed to different conditions including BPS, isoproterenol, BPS plus isoproterenol or BPA plus isoproterenol for 30 minutes as ECG was continuously recorded. The electrical rhythm of heart was read from the recorded electrocardiograph, and the number of arrhythmic events within the treatment time, including

ventricular tachycardia and premature ventricular beats were counted. A total of 25 female hearts and 20 male hearts were used as 45 preparations in this experiment.

Isolation of rat ventricular myocytes

Ventricular myocytes were enzymatically dissociated from rat hearts using Langendorff perfusion as previously described (Yan et al. 2011). Briefly, the hearts were perfused with a Tyrode's solution containing 0.7 mg/ml type II collagenase (Worthington Biochem). Following digestion the ventricles were minced and myocytes were suspended in Tyrode's solution. For immunoblotting, myocytes were cultured on laminin-coated polystyrene culture dishes in a Medium-199 based solution (Gibco; catalog number 31100-035, with Earle's salts and L-glutamine) at 37 ^{o}C for 4 hours prior to treatment and collection.

Immunoblotting

Immunoblotting was performed as previously described (Gao et al. 2013). Isolated ventricular myocytes were subject to different treatments including control and 5 min exposure to 10^{-9} M BPS, 10^{-9} M BPS + 10^{-6} M MPP, 10^{-9} M BPS + 5 x 10^{-6} M PHTPP. Treatments were ended with aspirating chemical-containing medium and washing with ice-cold PBS solution. After centrifuging, sedimented myocytes were snap-frozen with liquid nitrogen. Equal amounts of protein extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked with 5% nonfat milk (supplemented with 0.1% Tween 20 in PBS) and incubated with primary antibodies and secondary antibodies as indicated. An enhanced chemiluminescence (ECL) Western blotting analysis system (GE

Healthcare) was used for developing the membrane. Antibodies used in the study are: pSer2808-RYR2, pSer2814-RYR2, pSer16-PLN, pThr17-PLN, PLN-A1, RYR2 (Badrilla); horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (Cell Signaling Technology). By using the phosphorylated protein-specific antibodies, the phosphorylated ryanodine receptor and phospholamban at specific sites, i.e., pSer2808-RYR2, pSer2814-RYR2, pSer16-PLN, pThr17-PLN, were detected as blotting bands. Level of total ryanodine receptor and phospholamban were detected using anti-RYR2 and anti-PLN-A1 antibodies, respectively. The densities of bands were compared between control condition and BPS treatment condition, also with BPS plus MPP or PHTPP treatments. 3 female hearts and 3 male hearts were used for myocyte protein extractions in this experiment.

Myocyte mechanics and triggered activity analysis and imaging of myocyte Ca^{2+} transient and spark

The acute effects of BPS or BPA on myocyte mechanics and Ca^{2+} handling were examined in vitro following rapid exposure of 1 to 7 minutes. Myocyte contraction and after-contraction were recorded as previously described (Yan et al. 2011). Contractility was imaged with a charge-coupled device camera and a video-edge detector (Crescent Electronics). The myocytes were excited under field electrical pacing, which was generated by filed stimulation with a Grass S48 stimulator (Grass instrument). Myocytes were paced at a frequency of 0.5 Hz, and with 2 ms pulses at an intensity of 1.5x the threshold for activation. Data were sampled through an Axon Digidata 1322A board using the PCLAMP 9 software (both by Molecular Devices). After-contraction was measured following electrical pacing at 2 Hz for 8 sec. Ca^{2+} spark was

recorded from myocytes loaded with fluo-4 acetoxymethyl ester (5 μM; Molecular Probes) with a Zeiss LSM 710 inverted confocal microscope at excitation wavelength of 488 nm. Fluorescent signals measured at > 515 nm in line-scan mode at 3.07 ms intervals, with each line comprising 512 pixels spaced at 0.056 mm. Ca^{2+} sparks are quantal and spontaneous release of Ca^{2+} from the sarcoplasmic reticulum through the RyRs, and were observed as localized and transient rises in fluorescent intensity under confocal imaging. Analysis of Ca^{2+} spark images were performed using IDL software (ITT Visual Information Solutions). Ca^{2+} spark frequency was calculated as the number of sparks divided by the length of scan line, divided by the time of scan, and expressed as n/100 μm/sec. Ca^{2+} spark amplitude was calculated as the peak signal intensity of the spark and expressed as peak fluorescent intensity of spark divided by baseline intensity (F/F_0). To measure Ca^{2+} transients, fluorescence signals were measured from fluo-4 acetoxymethyl ester loaded myocytes using a Nikon TE 2000 microscope and an InCyt Standard PM photometry system (Intracellular Imaging). Data analysis and image processing were performed as previously described (Yan et al. 2011). A total of 17 female hearts and 7 male hearts were used as sources of isolated ventricular myocytes in these experiments.

Statistical analysis

All experiments were repeated independently using myocytes isolated from at least 3 rat hearts. One-way analysis of variance (ANOVA) with a multiple comparison post-hoc test or t-test was conducted to compare differences between treatment groups. Chi-square (χ^2) test was used to analyze frequency of events (e.g., percentage of myocytes with triggered activities). Minimal

level of statistical significance for differences in values is considered to be $P < 0.05$. Data was analyzed with SigmaPlot 11.0 and expressed as average ± SEM.

Results

Effects of BPS on the electrical activities of *ex vivo* female rat hearts

The rapid impact of BPS on the electrical rhythm of *ex vivo* female rat hearts was examined using surface electrocardiography (Fig. 2). All hearts showed normal sinus rhythms under control condition. Exposed to 10^{-9} M BPS did not trigger any detectable arrhythmia events (Fig. 2A and 2C), but resulted in a moderate increase in heart rates from 290 to 319 beats/min ($P < 0.05$; Fig. 2A and 2B).

Under stress condition created with the β-adrenergic agonist isoproterenol (Iso; 10^{-8} M), 10^{-9} M BPS markedly increased the frequency of premature ventricular beats (PVBs; Fig. 2D and 2C, red asterisks indicate PVBs), from 0.87 events/20 min under baseline (i.e., Iso alone) to 8.83 events/20 min under BPS plus Iso ($P < 0.05$). Under stress condition, the pro-arrhythmic effect of BPS was comparable to that of 10^{-9} M BPA; under BPA plus Iso the frequency of PVBs was 9.00 events/20 min (Fig. 2C). In the presence of Iso, BPS also triggered episodes of non-sustained ventricular tachycardia (VT) in 1 out of 6 female rat hearts (Fig. 2D). Non-sustained VT was not observed in any hearts under Iso alone.

Effects of BPS on triggered activities in female rat ventricular myocytes

The effect of BPS on the development of spontaneous excitations in female rat ventricular myocytes was examined. These aberrant excitations, recorded as spontaneous Ca^{2+} transients

following repeatedly pacing, are known as "triggered activities" (Bers 2002). Acute exposure to 10^{-9} M BPS for 2-7 min resulted in triggered activities in 30% of female rat ventricular myocytes (Fig. 2E and 2F); by comparison, under control condition triggered activities were observed in 6.6% of myocytes ($P < 0.05$). The stimulatory effect of 10^{-9} M BPS on triggered activity was similar to that of 10^{-9} M BPA (Fig. 2F). Unlike the robust effect seen at 10^{-9} M, 10^{-12} M or 10^{-6} M BPS did not alter the percentage of cells with triggered activities, giving rise to an inverted-U shaped dose response (Fig. 2F).

Effects of BPS on Ca^{2+} handling in female rat ventricular myocytes

Myocyte Ca^{2+} handling is fundamental to cardiac physiology; abnormal Ca^{2+} handling is a key mechanism of arrhythmogenesis (Bers 2002). Previously we showed that alteration of myocyte Ca^{2+} handling plays an important role in the pro-arrhythmic action of BPA (Yan et al. 2011). The acute effects of BPS (10^{-9} M) on Ca^{2+} handling in female rat ventricular myocytes were examined. BPS rapidly increased the amplitude of field-stimulated Ca^{2+} transient (Fig. 3A), from 1.88 (F/F_0 ratio) under control to 4.80 under BPS (Fig. 3B). BPS also significantly decreased the time constant of field-stimulated Ca^{2+} transient (595.0 ms under control vs. 386.3 ms under BPS; Fig. 3B), indicating an increase in sarcoplasmic reticulum (SR) Ca^{2+} reuptake. Increased diastolic SR Ca^{2+} release, or Ca^{2+} leak, plays a key role in the arrhythmogenic effect of BPA in the heart (Gao et al. 2013). SR Ca^{2+} release was assessed as Ca^{2+} sparks in quiescent female rat ventricular myocytes. Acute exposure to 10^{-9} M BPS significantly increased Ca^{2+} spark frequency, from 1.36 to 2.18 sparks/s/100 μm (Fig. 3C and 3D), without altering Ca^{2+} spark peak

amplitude (Fig. 3E) or temporal/spatial properties (not shown). Blockade of estrogen receptor (ER) β with selective blocker PHTPP completely abolished BPS' effects on Ca^{2+} spark in female rat ventricular myocytes (Fig. 3C and 3D). PHTPP alone in the absence of BPS had no detectable effect on Ca^{2+} sparks in female ventricular myocytes (Fig. 3D and 3E).

Myocyte contraction is a manifestation of the Ca^{2+} cycling process, and was used as a global index to further assess the effects of BPS. BPS enhanced fractional shortening of female ventricular myocytes in a dose-dependent manner with an inverted-U shaped dose response curve (Fig. 3F). The most efficacious effect of BPS (at 10^{-9} and 10^{-8} M) was comparable to the effect of 10^{-9} M BPA. Combination of BPS and BPA (10^{-9} M + 10^{-9} M) did not produce any increase in effectiveness, indicating no synergistic or antagonistic actions (Fig. 3G). Selective blockade of ERβ with PHTPP, but not ERα with MPP, abolished the effects of BPS on myocyte contractility (Fig. 2G).

Effects of BPS on phosphorylation status of Ca^{2+} handling proteins in female rat ventricular myocytes

Ryanodine receptor (RyR) and phospholamban (PLN) are two key Ca^{2+} handling/regulatory proteins that can be phosphorylated to modify SR release and reuptake of Ca^{2+} (Kranias and Hajjar 2012; Van Petegem 2012). The effects of BPS on the phosphorylation status of RyR and PLN were examined by western blot in female rat ventricular myocytes (Fig. 4). Similar to the effects of BPA (Gao et al. 2013), 10^{-9} M BPS rapidly and transiently increased phosphorylation of RyR at the protein kinase A (PKA) site serine 2808 (Fig. 4A), and PLN at the

Ca^{2+}/CaM-dependent protein kinase II (CAMKII) site threonine 17 (Fig. 4C). The effects of BPS on RyR and PLN phosphorylation were detectable upon 30 s of exposure and peaked at 5 min. BPS did not affect the phosphorylation of RyR at the CAMKII site (serine 2814; Fig. 4B) or PLN at the PKA site (serine 16; Fig. 4D). Selective blockade of ERβ with PHTPP, but not ERα with MPP, completely abolished the effects of BPS on the phosphorylation status of RyR as well as PLN (Fig. 4E and 4F). PHTPP alone had no detectable effect on phosphorylation of RyR or PLN (not shown).

Responses to BPS in male rat hearts and ventricular myocytes

In striking contrast to the pro-arrhythmic effects observed in female heart, BPS did not have any detectable rapid action in male rat hearts. At the male whole heart level, the frequency of ectopic beats under 10^{-9} M BPS plus Iso was similar to that under Iso alone ($P > 0.3$) and 10^{-9} M BPS had no effect on the heart rate (Fig. 5A and 5B). At the cellular level, BPS did not increase the incidence of triggered activities in male cardiac myocytes (Fig. 5C), or alter the frequency of Ca^{2+} sparks (Fig. 5D) or the kinetics of Ca^{2+} transient (Fig. 5E). At the protein level, rapid exposure to BPS did not alter the phosphorylation levels of RyR by PKA or CAMKII (Fig. 5F and 5G), or of PLN by PKA or CAMKII (Fig. 5H and 5I).

Using incidence of triggered activity and PKA phosphorylation of RyR serine 2808 as endpoints, we examined the mechanism underlying the lack of response of male myocytes to BPS (Fig. 6). While BPS did not increase the incidence of triggered activities (Fig. 6A) or RyR phosphorylation (Fig. 6B), activation of ERβ with selective agonist DPN had marked stimulatory

effects on these endpoints. Interestingly, the stimulatory effects of BPS were revealed under ERα blockade with MPP (while MPP alone had no measurable effects) (Fig. 6A and 6B).

Discussion

With recognition of the potential adverse health impact of BPA exposure, BPS, a member of the bisphenol family with a structure similar to that of BPA, is increasingly been used as a BPA alternative in the production of consumer goods. Recent study showed that BPS is becoming a common environmental chemical (Liao and Kannan 2013). However, unlike the extensive studies of BPA, current knowledge on the biological and potential toxicological impact of BPS and other BPA substitutes is limited. In the present study, we report that low-dose BPS had rapid impact in female rat hearts at the organ, cellular and protein levels. These actions were female-specific, and have striking parallel to the pro-arrhythmic cardiac effects of equal dose BPA (Yan et al. 2011). These results demonstrate that BPS, at environmentally-relevant dose, may also have potential adverse effects on female hearts, and suggest that BPS, and possibly other BPA substitutes, is not necessarily free of the adverse health effects associated with BPA.

Limited but growing evidence suggests that BPS has potential estrogenic endocrine disrupting activities. BPS, mostly at supra-physiological doses (μM to mM), was found to have genomic estrogenic activities in heterologous cell lines (Grignard et al. 2012; Hashimoto et al. 2001; Kuruto-Niwa et al. 2005). Recent studies demonstrated that BPS exposure had adverse effects on reproduction and progeny generation in zebrafish at nM dose range (Ji et al. 2013), and altered

progesterone, testosterone and cortisol synthesis in human adrenal cortico-carcinoma cells (Rosenmai et al. 2014). Acute exposure to low-dose BPS was shown to activate ERK rapid signaling in a pituitary cell line (Vinas and Watson 2013). In addition, it was shown that exposure to BPS for three consecutive days, at 20 and 500 mg/kg dose, increased the uterine weight in female rats (Yamasaki et al. 2004).

Here, we report that at the myocyte level, nM concentration BPS rapidly increased the incidence of aberrant spontaneous excitation in female myocytes. These "triggered activities" are single cell arrhythmia events that can propagate in the myocardium under certain pathophysiological conditions, and are well recognized as one of the central arrhythmogenic mechanisms in the heart (Bers 2002). At the whole heart level, exposure to BPS alone resulted in a moderate increase in heart rate, but did not trigger any arrhythmia events. Previously we showed that BPA resulted in rapid increase of intracellular cAMP level in female rat ventricular myocytes (Gao et al. 2013). The pacemaker current, and consequently the rate of automaticity of cardiac nodal cells, are subject to regulation by cAMP, both directly and through PKA (Baruscotti and Difrancesco 2004). Although the impact of BPS on pacemaker cells has not been investigated in the present study, it is possible that a cAMP-mediated mechanism is responsible for the effect of BPS on heart rate. The effect of BPS on cardiac rhythm was also examined under catecholamine-induced stress condition. β-adrenergic stimulation increases Ca^{2+} influx and SR Ca^{2+} load, and favor SR overload and abnormal Ca^{2+} release, thereby providing a substrate that favors arrhythmogenesis (Bers 2002). Under such stress condition, BPS exposure triggered

frequent premature ventricular beats, one of the most common forms of ventricular arrhythmias; in parallel comparisons, the effects of 10^{-9} M BPS on incidence of ectopic ventricular beats were indistinguishable to those of 10^{-9} M BPA. These results suggest that while BPS exposure by itself does not trigger clinically relevant arrhythmia events in healthy hearts under normal conditions, it may contribute to the development of arrhythmias in the presence of existing arrhythmogenic substrate such as elevated sympathetic tone and cardiac diseases.

The mechanism of the actions of BPS in female cardiac myocytes appears to be similar to that of BPA, and involved rapid alteration of myocyte Ca^{2+} handling. BPS significantly and rapidly increased SR Ca^{2+} reuptake, and importantly, diastolic SR Ca^{2+} release, or Ca^{2+} leak, a pathophysiological change that plays a key role in arrhythmogenesis in cardiac diseases (Chelu and Wehrens 2007). In previous study we showed that suppression of SR Ca^{2+} leak blocked the BPA-induced triggered activities in female cardiac myocytes (Yan et al. 2011). The impact of BPS on myocyte Ca^{2+} handling was mediated by characteristic impact on the phosphorylation of two key Ca^{2+} handling proteins, RyR and PLN (Gao et al. 2013). A nearly identical impact was also observed for BPS. RyR is the Ca^{2+} release channel of SR in cardiac myocytes, and its activity is subject to regulation by kinase phosphorylation. The two major phosphorylation sites are serine 2808 by PKA and serine 2814 by CAMKII. Either site's phosphorylation by individual kinase increases RyR opening probability via decreasing the threshold of Ca^{2+} sensor (Van Petegem 2012). PLN, on the other hand, regulates the Ca^{2+} reuptake process by its inhibitory interaction with sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Kranias and Hajjar 2012).

PLN also has two major phosphorylation sites, serine 16 (PKA) and threonine 17 (CAMKII). Phosphorylation of either site releases PLN's inhibition of SERCA, thereby increasing Ca^{2+} reuptake into the SR (Kranias and Hajjar 2012). We showed that BPS exposure rapidly and transiently increased phosphorylation of RyR by PKA but not by CAMKII, and PLN by CAMKII but not PKA. The time course of action of BPS and the target-specific manner of BPS' effect are remarkably similar to those described for the cardiac actions of BPA (Gao et al. 2013). Upstream, we showed that the impact of BPA on RyR and SR Ca^{2+} release is mediated by the adenylyl cyclase-cAMP-PKA signaling pathway, and the impact on PLN is mediated by the phospholipase C-inositol trisphosphate receptor-CAMKII pathway (Gao et al. 2013). A similar upstream signaling mechanism may also mediate the rapid actions of BPS.

With respect to the pharmacodynamics of BPS' action, in heterologous expression systems BPS was shown to have similar potency (EC_{50}) as BPA in activating an estrogen-responsive GFP reporter gene (Kuruto-Niwa et al. 2005); yet in a more recent study it was demonstrated that BPS was >20-fold less potent than BPA in activating an estrogen-responsive luciferase reporter gene, less potent in activating ERα and ERβ-driven luciferase activities, and had lower affinity in binding to ERα and ERβ in a competitive binding assay (Molina-Molina et al. 2013). BPS was also found to have anti-androgen effects with a lower potency compared with BPA (Kitamura et al. 2005). Here, we show that the pharmacodynamic properties of BPS' rapid action in cardiac myocytes closely resembled those of BPA. Using incidence of arrhythmogenic triggered activity and myocyte mechanics as endpoints, the dose response curve of rapid effects of BPS had a

nonmonotonic shape, similar to that described for BPA (Liang et al. 2014). The two chemicals have similar potency and efficacy, and the same most efficacious dose around 10^{-9} to 10^{-8} M. These findings contrast with the lower potency of BPS compared with BPA reported in other studies (Kitamura et al. 2005; Molina-Molina et al. 2013). Nonmonotonic dose responses are commonly observed for hormones and EDCs, and have important implication for the assessment of toxicity of EDCs (Vandenberg et al. 2012b). Previously, we demonstrate that the nonmonotonic dose response of BPA is the result of multiple monotonic actions on individual elements of myocyte Ca^{2+} handling processes, and the combined effect of its stimulatory effect on SR Ca^{2+} release and reuptake and the opposing inhibitory effect on Ca^{2+} influx through the L-type Ca^{2+} channel (Liang et al. 2014). These findings may provide clue on the mechanism giving rise to the nonmonotonic dose response of BPS in the heart.

There is striking contrast in the responses of female and male rat hearts to rapid low-dose BPS exposure. While female hearts responded robustly to BPS at the organ, cellular and protein levels, no detectable responses were found in male hearts when measured using those same endpoints. Importantly, BPS produced no pro-arrhythmic effects, including increased ectopic beats or ventricular tachycardia, in male whole hearts under Iso-induced stress, and did not increase the incidence of arrhythmic activities in isolated male myocytes. At the cellular and protein levels, BPS exposure rapidly altered myocyte SR Ca^{2+} handling and phosphorylation status of key Ca^{2+} handling proteins; these effects were absent in male rat hearts. In previous studies, we demonstrated that such remarkable sex-specific cardiac response to estrogenic

chemicals is due to the opposing and counterbalancing actions of ER signaling (Belcher et al. 2012). We showed that in both male and female rat cardiac myocytes, ERβ signaling had stimulatory effects on Ca^{2+} handling and development of triggered activity, while ERα signaling was inhibitory. The response of the heart was determined by the balance of ERβ and ERα signaling. Thus, the pro-arrhythmic effect of estrogenic chemicals in female rodent heart was dominated by the stimulatory ERβ rapid signaling, while in male hearts the effect of ERβ was present but masked by the inhibitory ERα signaling, resulting in a lack of observable response. Results shown in Figure 6 suggest that a similar mechanism mediates the lack of response of male cardiac myocytes to BPS. Consistent with this model, activation of ERβ alone with DPN revealed that the stimulatory effect of ERβ signaling was intact in male cells, and in the presence of ERα blockade the stimulatory effect of BPS (presumably via ERβ) was then observed.

In conclusion, we demonstrated that rapid exposure to low-dose BPS has pro-arrhythmic impact on female rat hearts through mechanisms involving activation of ERβ signaling and alteration of myocyte Ca^{2+} handling. The cardiac actions of BPS, as measured by multiple endpoints, are similar to those previously reported for BPA; also similar are the cellular and molecular mechanisms underlying the effects of the two chemicals. As a case study, our findings suggest that BPS and other structurally related BPA substitutes may share similar endocrine disrupting activities as BPA.

References

Baruscotti M, Difrancesco D. 2004. Pacemaker channels. Ann N Y Acad Sci 1015:111-121.

Belcher SM, Chen Y, Yan S, Wang H-S. 2012. Rapid estrogen receptor-mediated mechanisms determine the sexually dimorphic sensitivity of ventricular myocytes to 17beta-estradiol and the environmental endocrine disruptor bisphenol A. Endocrinology 153:712-720.

Bers DM. 2002. Cardiac excitation-contraction coupling. Nature 415:198-205.

Chelu MG, Wehrens XH. 2007. Sarcoplasmic reticulum calcium leak and cardiac arrhythmias. Biochem Soc Trans 35:952-956.

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, et al. 2009. Endocrine-disrupting chemicals: An Endocrine Society scientific statement. Endocr Rev 30:293-342.

European Commission. 2011. Bisphenol A: Eu ban on baby bottles to enter into force tomorrow. http://europaeu/rapid/press-release_IP-11-664_enhtm

Gao X, Liang Q, Chen Y, Wang H-S. 2013. Molecular mechanisms underlying the rapid arrhythmogenic action of bisphenol A in female rat hearts. Endocrinology 154:4607-4617.

Grignard E, Lapenna S, Bremer S. 2012. Weak estrogenic transcriptional activities of bisphenol A and bisphenol S. Toxicol in Vitro 26:727-731.

Hashimoto Y, Moriguchi Y, Oshima H, Kawaguchi M, Miyazaki K, Nakamura M. 2001. Measurement of estrogenic activity of chemicals for the development of new dental polymers. Toxicol in Vitro 15:421-425.

Ji K, Hong S, Kho Y, Choi K. 2013. Effects of bisphenol S exposure on endocrine functions and reproduction of zebrafish. Environ Sci Technol 47:8793-8800.

Kitamura S, Suzuki T, Sanoh S, Kohta R, Jinno N, Sugihara K, et al. 2005. Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. Toxicological Sciences 84:249-259.

Kranias EG, Hajjar RJ. 2012. Modulation of cardiac contractility by the phopholamban/SERCA2A regulatome. Circ Res 110:1646-1660.

Kuruto-Niwa R, Nozawa R, Miyakoshi T, Shiozawa T, Terao Y. 2005. Estrogenic activity of alkylphenols, bisphenol S, and their chlorinated derivatives using a GFP expression system. Environ Toxicol Phar 19:121-130.

LaKind JS, Goodman M, Naiman DQ. 2012. Use of NHANES data to link chemical exposures to chronic diseases: A cautionary tale. PLoS One 7:e51086.

Lang IA, Galloway TS, Scarlett A, Henley WE, Depledge M, Wallace RB, et al. 2008. Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults. JAMA 300:1303-1310.

Legifrance.gouv.fr. 2012. Visant à la suspension de la fabrication, de l'importation, de l'exportation et de la mise sur le marché de tout conditionnement à vocation alimentaire contenant du bisphénol A http://wwwlegifrancegouvfr/affichTextedo?cidTexte=JORFTEXT000026830015

Liang Q, Gao X, Chen Y, Hong K, Wang H-S. 2014. Cellular mechanism of the nonmonotonic dose response of bisphenol A in rat cardiac myocytes. Environ Health Perspect 122:601-608.

Liao C, Liu F, Alomirah H, Loi VD, Mohd MA, Moon HB, et al. 2012a. Bisphenol S in urine from the United States and seven Asian countries: Occurrence and human exposures. Environ Sci Technol 46:6860-6866.

Liao C, Liu F, Kannan K. 2012b. Bisphenol S, a new bisphenol analogue, in paper products and currency bills and its association with bisphenol A residues. Environ Sci Technol 46:6515-6522.

Liao CY, Kannan K. 2013. Concentrations and profiles of bisphenol A and other bisphenol analogues in foodstuffs from the United States and their implications for human exposure. J Agr Food Chem 61:4655-4662.

Melzer D, Rice NE, Lewis C, Henley WE, Galloway TS. 2010. Association of urinary bisphenol A concentration with heart disease: Evidence from NHANES 2003/06. PLoS One 5:e8673.

Melzer D, Osborne NJ, Henley WE, Cipelli R, Young A, Money C, et al. 2012. Urinary bisphenol A concentration and risk of future coronary artery disease in apparently healthy men and women. Circulation 125:1482-1490.

Molina-Molina JM, Amaya E, Grimaldi M, Saenz JM, Real M, Fernandez MF, et al. 2013. In vitro study on the agonistic and antagonistic activities of bisphenol S and other bisphenol A congeners and derivatives via nuclear receptors. Toxicol Appl Pharmacol 272:127-136.

Rosenmai AK, Dybdahl M, Pedersen M, Alice van Vugt-Lussenburg BM, Wedebye EB, Taxvig C, et al. 2014. Are structural analogues to bisphenol A safe alternatives? Toxicol Sci 139:35-47.

US Food and Drug Administration. 2012. Indirect food additives: Polymers. Fed Reg 77:41899-41902.

Van Petegem F. 2012. Ryanodine receptors: Structure and function. J Biol Chem 287:31624-31632.

Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. 2007. Human exposure to bisphenol A (BPA). Reprod Toxicol 24:139-177.

Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJ, Schoenfelder G. 2012a. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. Cien Saude Colet 17:407-434.

Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Jr., Lee DH, et al. 2012b. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocr Rev 33:378-455.

Vinas P, Campillo N, Martinez-Castillo N, Hernandez-Cordoba M. 2010. Comparison of two derivatization-based methods for solid-phase microextraction-gas chromatography-mass spectrometric determination of bisphenol A, bisphenol S and biphenol migrated from food cans. Anal Bioanal Chem 397:115-125.

Vinas R, Watson CS. 2013. Bisphenol S disrupts estradiol-induced nongenomic signaling in a rat pituitary cell line: Effects on cell functions. Environ Health Perspect 121:352-358.

Yamasaki K, Noda S, Imatanaka N, Yakabe Y. 2004. Comparative study of the uterotrophic potency of 14 chemicals in a uterotrophic assay and their receptor-binding affinity. Toxicol Lett 146:111-120.

Yan S, Chen Y, Dong M, Song W, Belcher SM, Wang H-S. 2011. Bisphenol A and 17beta-estradiol promote arrhythmia in the female heart via alteration of calcium handling. PLoS One 6:e25455.

Yan S, Song W, Chen Y, Hong K, Rubinstein J, Wang H-S. 2013. Low-dose bisphenol A and estrogen increase ventricular arrhythmias following ischemia-reperfusion in female rat hearts. Food Chem Toxicol 56C:75-80.

Ye XB, Pierik FH, Hauser R, Duty S, Angerer J, Park MM, et al. 2008. Urinary metabolite concentrations of organophosphorous pesticides, bisphenol A, and phthalates among pregnant women in Rotterdam, the Netherlands: The Generation Rstudy. Environ Res 108:260-267.

Zoeller RT, Brown TR, Doan LL, Gore AC, Skakkebaek NE, Soto AM, et al. 2012. Endocrine-disrupting chemicals and public health protection: A statement of principles from the Endocrine Society. Endocrinology 153:4097-4110.

Figure Legends

Figure 1. Structures of bisphenol S (BPS) and bisphenol A (BPA).

Figure 2. Effects of BPS exposure on female rat hearts and ventricular myocytes. (*A*) Left, surface ECG recording traces from female rat hearts under control and in the presence of 10^{-9} M BPS. Right, overlay of the same traces to show increased heart rate in the presence of 10^{-9} M BPS. R-waves in the presence of BPS are indicated by red arrows. Change in heart rate was observed within 5 minutes of BPS exposure. (*B*) Average heart rate of female rat hearts under control and in the presence of 10^{-9} M BPS. N = 3 hearts for each group. (*C*) Average frequency of premature ventricular beats (PVBs) in female rat hearts under indicated conditions. N = 6 - 8 hearts. (*D*) Example surface ECG traces from female rat hearts under 10^{-8} M isoproterenol (Iso) alone and in the presence of 10^{-9} M BPS and Iso. Red asterisks indicate PVBs. VT: ventricular tachycardia. (*E*) Example confocal images showing Ca^{2+} transients in female rat ventricular myocytes elicited by pacing under control and in the presence of 10^{-9} M BPS. Red arrows indicate spontaneous Ca^{2+} after transients (i.e., triggered activities) follow electrical pacing. (*F*) Percentage of female rat ventricular myocytes with triggered activities under various conditions. N = 30 myocytes for each group. *: $P < 0.05$ vs. control in a paired t-test (*B*), one-way ANOVA (*C*), or χ^2 test (*F*). Error bars are SEM.

Figure 3. Effects of BPS on Ca^{2+} handling in female rat ventricular myocytes. (*A*) Ca^{2+} transient traces in female rat ventricular myocytes elicited by field electrical stimulation under control and in the presence of 10^{-9} M BPS. (*B*) Average Ca^{2+} transient (CaT) amplitude and decay time constant (tau) under control and in the presence of 10^{-9} M BPS. N = 6 - 8 myocytes. (*C*) Example confocal images of Ca^{2+} sparks in female rat ventricular myocytes under control and in the presence of 10^{-9} M BPS and 10^{-9} M BPS + 5 x 10^{-6} M PHTPP. (*D*) and (*E*) are averages of Ca^{2+} spark frequency and amplitude under control, and in the presence of 10^{-9} M BPS, 10^{-9} M BPS + 5 x 10^{-6} M PHTPP and 5 x 10^{-6} M PHTPP alone. N = 6 - 7 myocytes. (*F*) Dose-dependent effect

of BPS on the contractility (mean fractional shortening) of female rat ventricular myocytes, and the effect of 10^{-9} M BPA. N = 25 - 26 myocytes. (*G*) Mean fractional shortening of female rat ventricular myocytes under indicated conditions. MPP: 10^{-6} M; PHTPP: 5 x 10^{-6} M. N = 11 - 12 myocytes. *: $P < 0.05$ vs. control in a one-way ANOVA test. N.S: not significant in a one-way ANOVA test. Error bars are SEM.

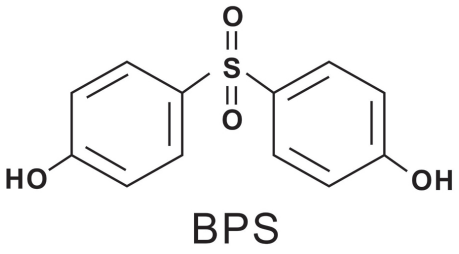
Figure 4. Effects of BPS on ryanodine receptor and phospholamban phosphorylation in female rat ventricular myocytes. (*A*) and (*B*) Immunoblots of phosphorylated RyR at serine 2808 (PKA site) and serine 2814 (CAMKII site), and total RyR from female rat ventricular myocytes under control and upon exposure to 10^{-9} M BPS for indicated time. Cont, control. (*C*) and (*D*) Immunoblots of phosphorylated PLN at threonine 17 (CAMKII site) and serine 16 (PKA site), and total PLN from female rat ventricular myocytes under control and upon exposure to 10^{-9} M BPS for indicated time. (*E*) Immunoblot of phosphorylated RyR at serine 2808 (PKA site) and total RyR from female rat ventricular myocytes under control and upon 5 min exposure to 10^{-9} M BPS, 10^{-9} M BPS + 10^{-6} M MPP, 10^{-9} M BPS + 5 x 10^{-6} M PHTPP. (*F*) Immunoblot of phosphorylated PLN at threonine 17 (CAMKII site) and total PLN from female rat ventricular myocytes under control and upon 5 min exposure to 10^{-9} M BPS, 10^{-9} M BPS + 10^{-6} M MPP, 10^{-9} M BPS + 5 x 10^{-6} M PHTPP.

Figure 5. Responses to BPS in male rat hearts and ventricular myocytes. (*A*) Example surface ECG traces from male rat hearts under 10^{-9} M BPS + 10^{-8} M isoproterenol. Red asterisk indicates PVB. (*B*) Left, average frequency of premature ventricular beats (PVBs) in male rat hearts under various conditions. N = 5 - 6 hearts. Right, average heart rate (beats per min) of male rat hearts under control and in the presence of 10^{-9} M BPS. N = 5 hearts for each group. (*C*) Percentage of male rat ventricular myocytes with triggered activities under indicated conditions. N = 42 - 43 myocytes. (*D*) Left, example confocal images of Ca^{2+} sparks in male rat ventricular myocytes under control and in the presence of 10^{-9} M BPS. Right, averages of Ca^{2+} spark frequency under control and in the presence of 10^{-9} M BPS. N = 10 myocytes for each group. (*E*) Average Ca^{2+}

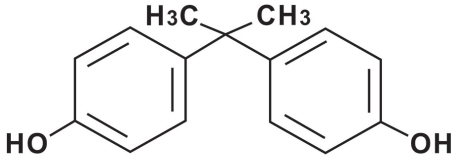
transient time constant under control and in the presence of 10^{-9} M BPS. N = 13 for each group. Error bars are SEM. N.S: not significant. (*F*) and (*G*) Immunoblots of phosphorylated RyR at serine 2808 (PKA site) and serine 2814 (CAMKII site), and total RyR from male rat ventricular myocytes under control and upon exposure to 10^{-9} M BPS for indicated time. Cont, control. (*H*) and (*I*) Immunoblots of phosphorylated PLN at threonine 17 (CAMKII site) and serine 16 (PKA site), and total PLN from male rat ventricular myocytes under control and upon exposure to 10^{-9} M BPS for indicated time.

Figure 6. (*A*) Percentage of male rat ventricular myocytes with triggered activities under indicated conditions. N = 24 myocytes for all groups. (*B*) Immunoblots of phosphorylated RyR at serine 2808 (PKA site) and total RyR from male rat ventricular myocytes under control and indicated treatments. Cont, control. Concentrations of drugs used were: BPS, 10^{-9} M; DPN, 10^{-7} M; MPP, 10^{-6} M.

Figure 1.



BPS



BPA

Figure 2.

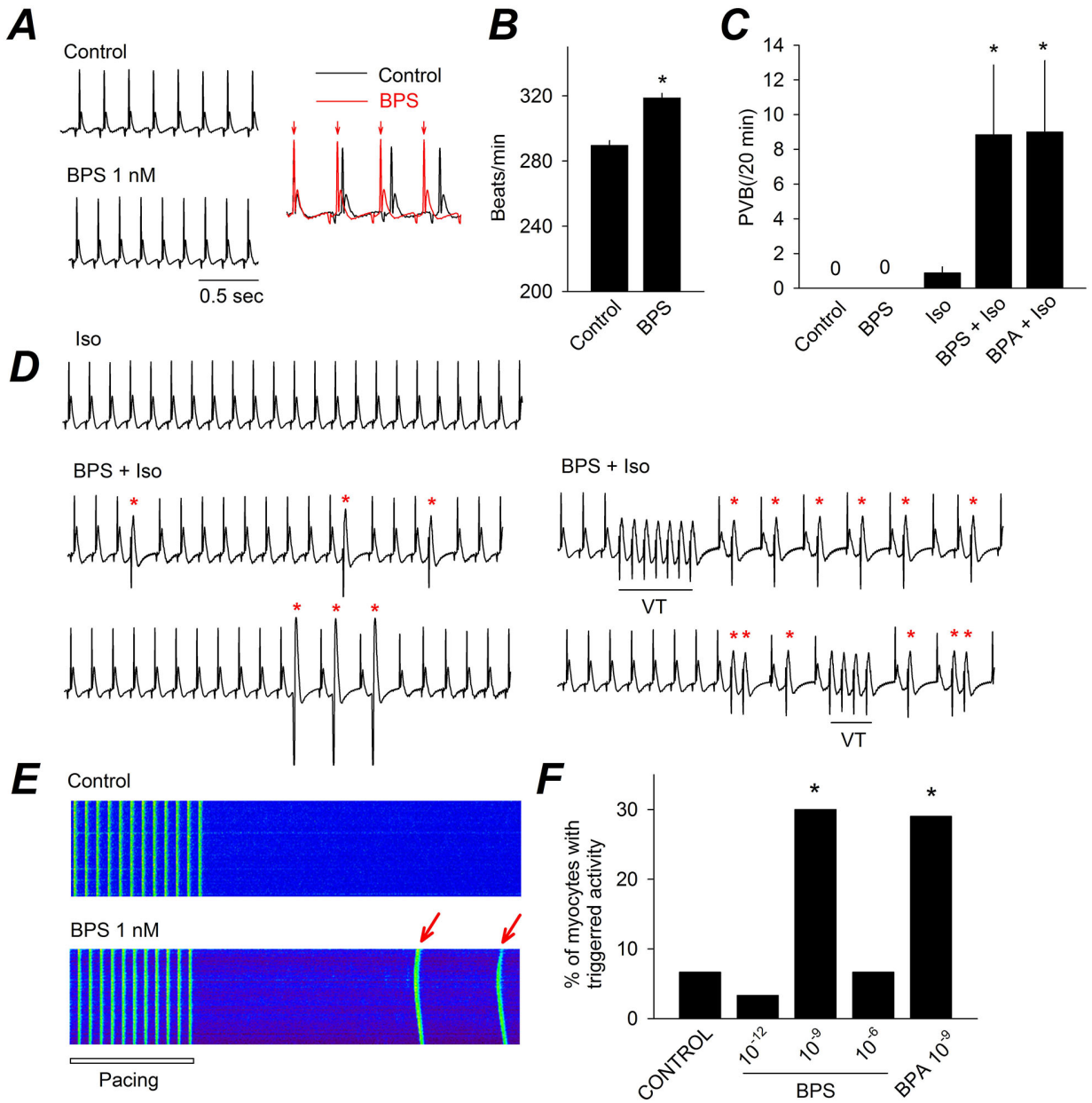

A
Control
BPS 1 nM
0.5 sec
Control
BPS
B
Beats/min
320
280
240
200
Control
BPS
C
PVB(/20 min)
14
12
10
8
6
4
2
0
0
0
Control
BPS
Iso
BPS + Iso
BPA + Iso
D
Iso
BPS + Iso
BPS + Iso
VT
VT
E
Control
BPS 1 nM
Pacing
F
% of myocytes with triggerred activity
30
20
10
0
CONTROL
10-12
10-9
10-6
BPS
BPA 10-9

Figure 3.

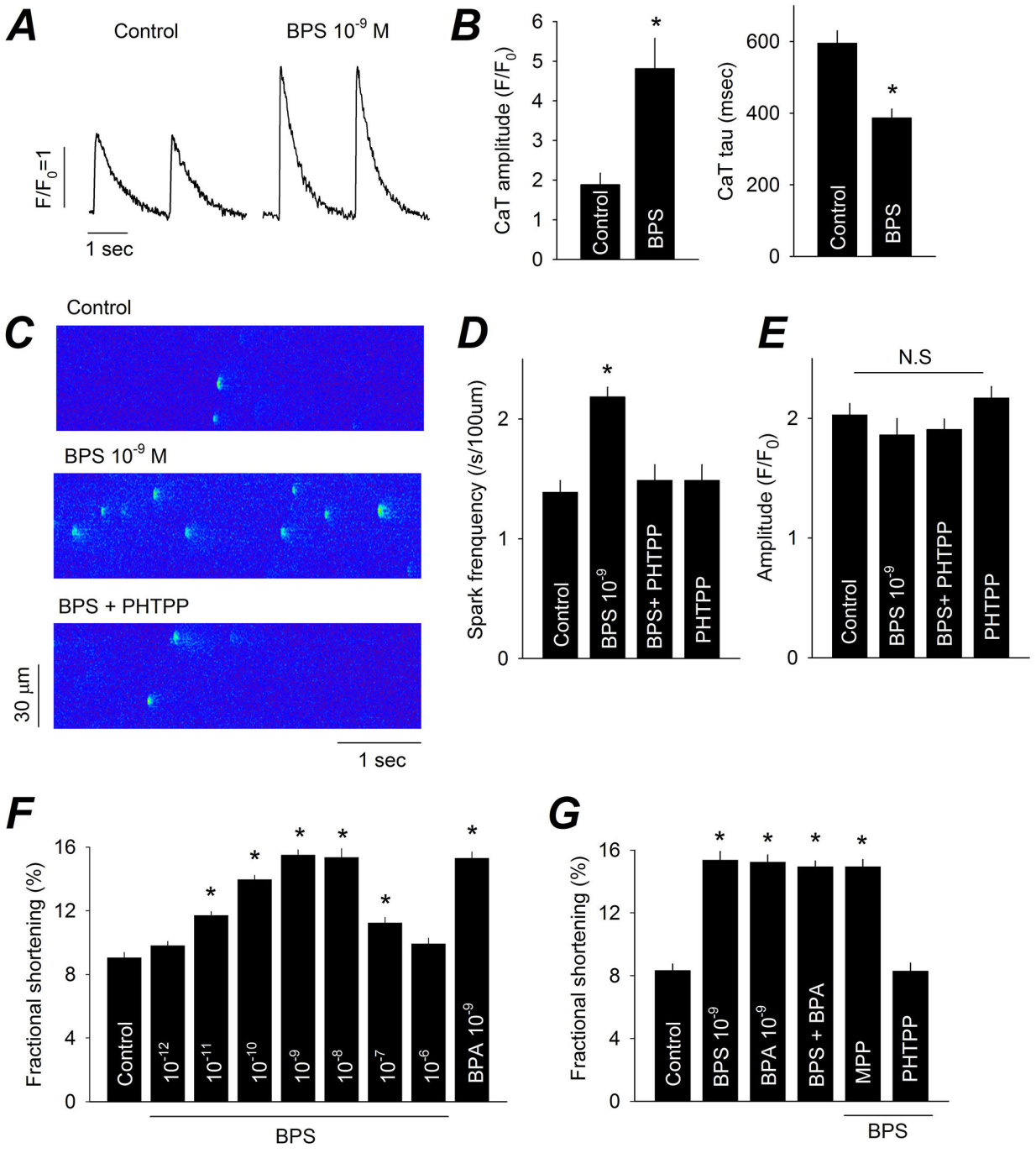

A
Control
BPS 10-9 M
F/F0=1
1 sec
B
CaT amplitude (F/F0)
Control
BPS
CaT tau (msec)
C
Control
BPS 10-9 M
BPS + PHTPP
30 µm
1 sec
D
Spark frenquency (/s/100um)
Control
BPS 10-9
BPS+ PHTPP
PHTPP
E
N.S
Amplitude (F/F0)
F
Fractional shortening (%)
Control
10-12
10-11
10-10
10-9
10-8
10-7
10-6
BPA 10-9
BPS
G
Fractional shortening (%)
BPS 10-9
BPA 10-9
BPS + BPA
MPP
PHTPP
BPS

Figure 4.

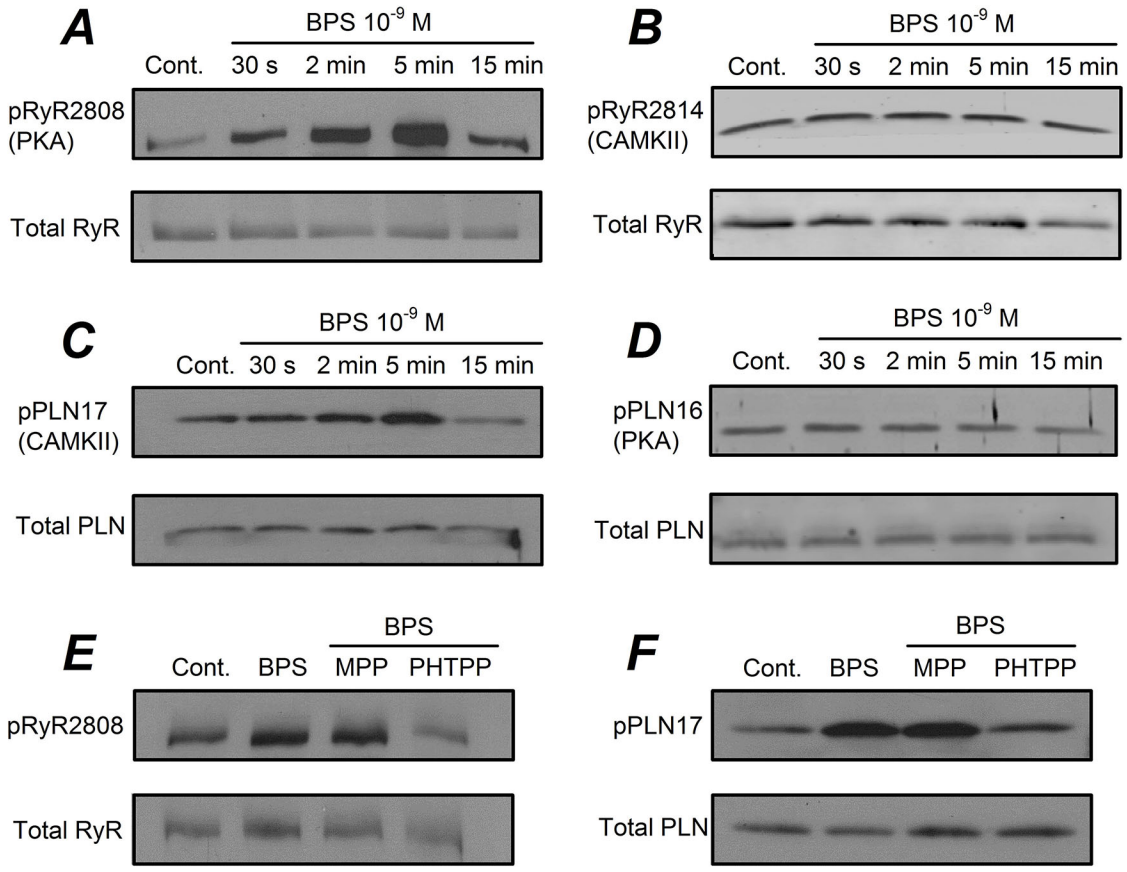

A
BPS 10-9 M
Cont.
30 s
2 min
5 min
15 min
pRyR2808 (PKA)
Total RyR
B
BPS 10-9 M
Cont.
30 s
2 min
5 min
15 min
pRyR2814 (CAMKII)
Total RyR
C
BPS 10-9 M
Cont.
30 s
2 min
5 min
15 min
pPLN17 (CAMKII)
Total PLN
D
BPS 10-9 M
Cont.
30 s
2 min
5 min
15 min
pPLN16 (PKA)
Total PLN
E
BPS
Cont.
BPS
MPP
PHTPP
pRyR2808
Total RyR
F
BPS
Cont.
BPS
MPP
PHTPP
pPLN17
Total PLN

Figure 5.

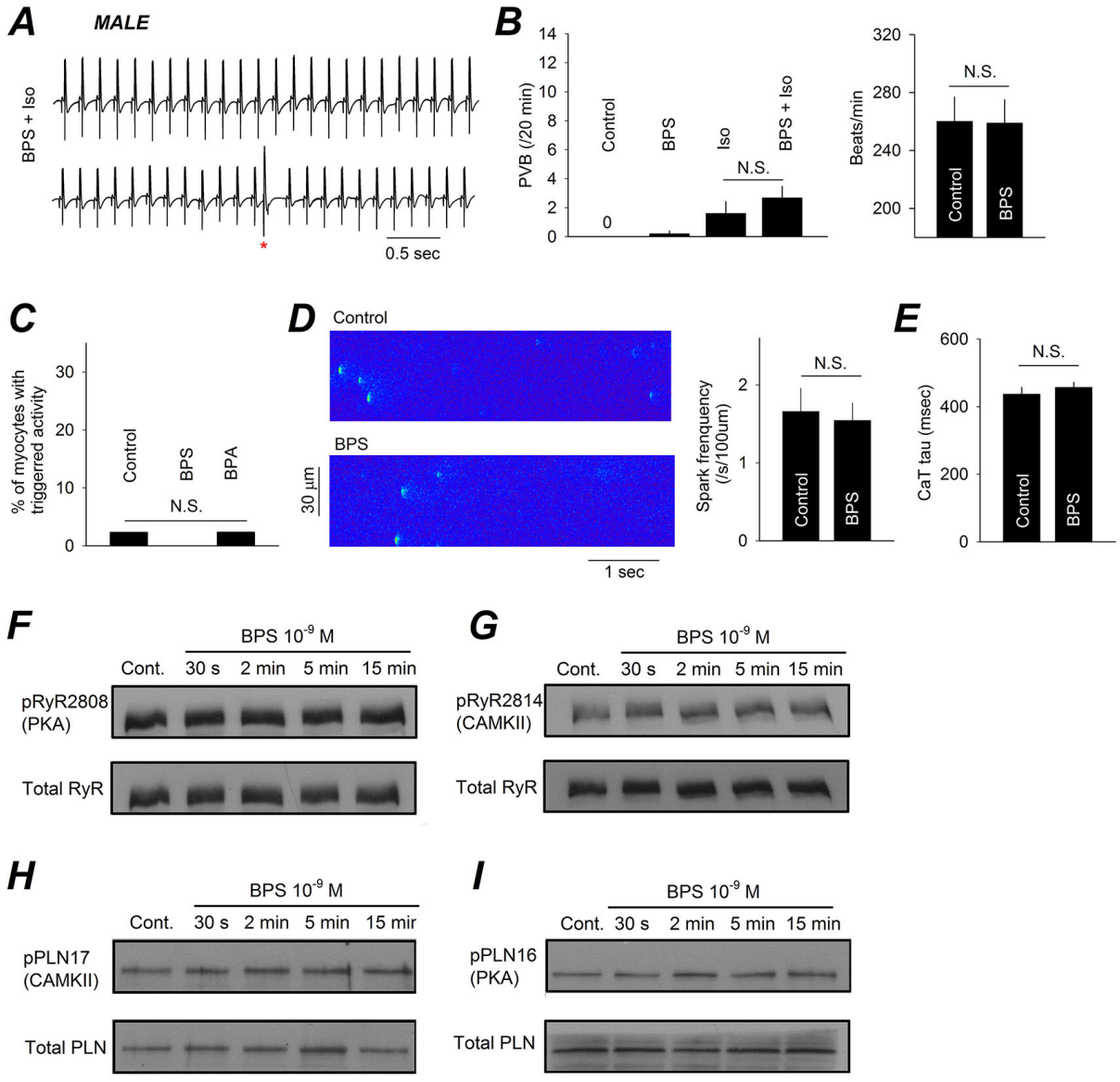
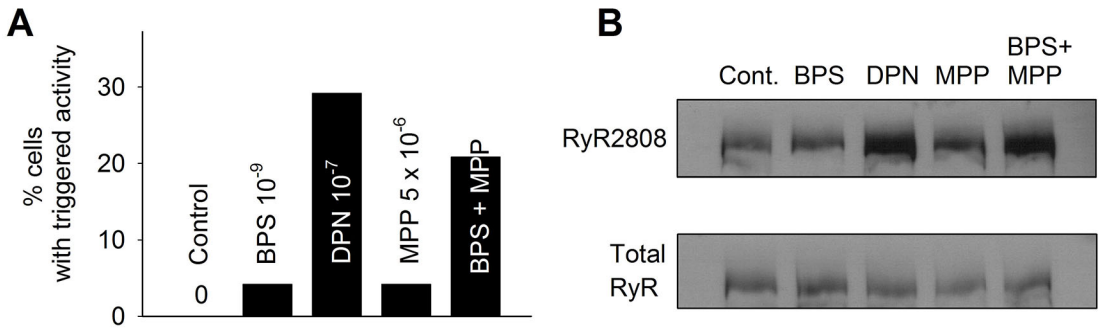

A
MALE
BPS + Iso
0.5 sec
B
PVB (/20 min)
Control
BPS
Iso
BPS + Iso
N.S.
Beats/min
Control
BPS
C
% of myocytes with triggerred activity
Control
BPS
BPA
N.S.
D
Control
BPS
30 μm
1 sec
Spark frenquency (/s/100um)
E
CaT tau (msec)
F
BPS 10-9 M
Cont. 30 s 2 min 5 min 15 min
pRyR2808 (PKA)
Total RyR
G
pRyR2814 (CAMKII)
H
pPLN17 (CAMKII)
Total PLN
I
pPLN16 (PKA)

Figure 6.


A
% cells
with triggered activity
30
20
10
0
Control
BPS 10-9
DPN 10-7
MPP 5 x 10-6
BPS + MPP
B
Cont.
BPS
DPN
MPP
BPS+
MPP
RyR2808
Total
RyR